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Short Article

Hedgehog and Bmp Polarize Hematopoietic Stem Cell Emergence in the Zebrafish Dorsal Aorta

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SUMMARY

Hematopoietic stem cells (HSCs) are first detected in the floor of the embryonic dorsal aorta (DA), and we investigate the signals that induce the HSC program there. We show that while continued Hedgehog (Hh) signaling from the overlying midline structures maintains the arterial program characteristic of the DA roof, a ventral Bmp4 signal induces the blood stem cell program in the DA floor. This patterning of the DA by Hh and Bmp is the mirror image of that in the neural tube, with Hh favoring dorsal rather than ventral cell types, and Bmp favoring ventral rather than dorsal. With the majority of current data supporting a model whereby HSCs derive from arterial endothelium, our data identify the signal driving this conversion. These findings are important for the study of the production of HSCs from embryonic stem cells and establish a paradigm for the development of adult stem cells.

INTRODUCTION

Blood and endothelium develop in close association, possibly from bipotential precursors called hemangioblasts (reviewed in Dzierzak and Speck, 2008). Bmp is critical for hemangioblast formation, and Hedgehog (Hh), VEGF, and Notch are required for the specification of arterial endothelium and adult HSCs in zebrafish (Gering and Patient, 2005; Lawson et al., 2002; McReynolds et al., 2007; Walmsley et al., 2002). Subsequent to its role in arterial and HSC specification, Hh is later required for continued expression of the transcription factor *tbx20* in the DA roof, proximal to the source of Hh in the notochord (Gering and Patient, 2005; Murayama et al., 2006; Zhang and Rodaway, 2007). This dorsal restriction of *tbx20* within the DA occurs at the same time as the initiation of definitive hematopoiesis in the ventral wall of the DA (Ahn et al., 2000; Gering and Patient, 2005) (Figure 1A), an observation which may reflect an antagonistic relationship between *tbx20* and the hematopoietic program, as seen during the earlier development of embryonic blood (Szeto et al., 2002). Thus, the restriction of HSC formation to the DA floor may be a consequence of continued Hh signaling.

While the location of Hh protein has not been determined in zebrafish due to a lack of available antibodies, *Hh* expression has been demonstrated within the dorsally located notochord, but not the ventrally located endoderm, during the time of HSC emergence (Krauss et al., 1993; Lewis et al., 1999; Roy et al., 2001). Perhaps the best readout for cells actively receiving Hh signaling is the expression of the receptor, Patched (Ptc), and *ptc1* is expressed within the dorsal wall of the DA, while *ptc2* exhibits more diffuse expression throughout the trunk, encompassing the entire DA (Lewis et al., 1999). The distinct functions of these receptors are not well understood, but it is highly likely that they will have different targets, based upon the disparate phenotypes observed in *ptc1* and *ptc2* mutants (Koudijs et al., 2005, 2008). Lewis et al.'s (1999) data indicate that *ptc1* expression requires a higher level of Hh signaling than *ptc2*, consistent with its expression in the dorsal wall of the DA, proximal to the source of midline Shh. Thus it is likely that Hh signaling in the dorsal wall of the DA is mediated by Ptc1. The expression of *ptc2* within the ventral wall of the DA, more distal to the midline Shh source, implies that the ventral DA experiences a low level of Hh signaling. However, inhibition of Hh signaling at this time indicated that it is not required for HSC programming (Gering and Patient, 2005).

Here we demonstrate that distance from Hh signaling contributes to, but is not solely responsible for, the ventral localization of HSCs. Using a conditional transgenic strategy and, in parallel, morpholino knockdown, we provide in vivo functional evidence that *bmp4* is required for the *runx1*-mediated emergence and maintenance of HSCs in the ventral wall of the DA.

RESULTS

Ectopic Hedgehog Signaling Expands *tbx20* Expression into the Ventral Wall of the DA but Does Not Suppress Initiation of HSC Gene Expression

Because *tbx20* and HSC gene expression are mutually exclusive, we tested the possibility that Hh might restrict HSC formation, by exposing the DA floor to continued Hh signaling. To

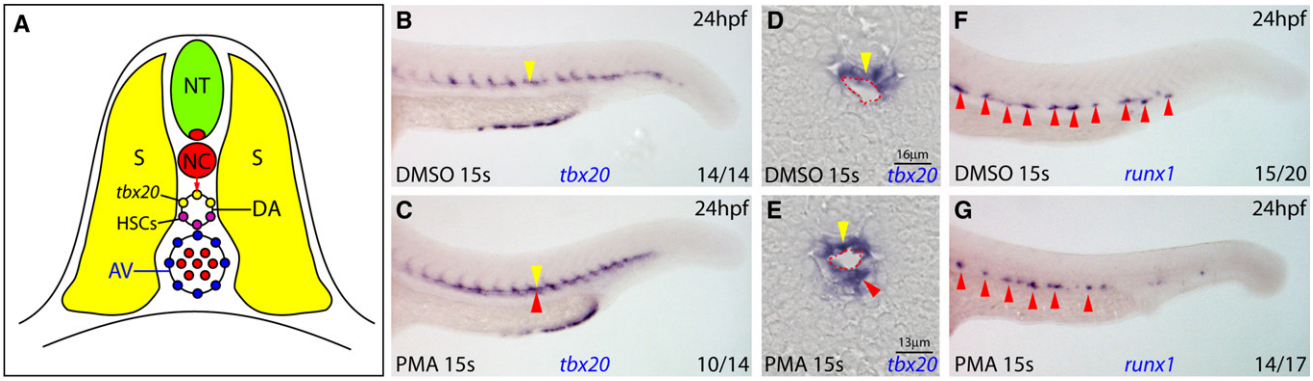

Figure 1. Ectopic Hedgehog Signaling Expands *tbx20* Expression into the Ventral DA but Does Not Suppress the Initiation of HSC Gene Expression

(A) Diagram of transverse section illustrating DA polarization in the zebrafish trunk at 24 hpf.

(B–G) Embryos treated with DMSO or PMA from 15S and probed for *tbx20* or *runx1* at 24 hpf. Dorsal and ventral DA expression (yellow and red arrows) are shown. PMA treatment expands *tbx20* expression into the ventral DA ([C] and [E] compared to [B] and [D]), but *runx1* is unaffected (F and G). AV, axial vein; DA, dorsal aorta; NC, notochord; NT, neural tube; PMA, purmorphamine; S, somite.

expand the range of Hh signaling, embryos were treated with purmorphamine (PMA), which activates the Smoothened receptor (Sinha and Chen, 2006). Treated embryos exhibited morphological and gene expression alterations that were characteristic of Hh pathway activation (see Figure S1 available online) (Concordet et al., 1996; Ekker et al., 1995). In embryos treated from the 15-somite stage, when maintenance of *tbx20* expression in the roof of the DA is Hh dependent, *tbx20* expression was expanded into the DA floor (Figures 1C and 1E, red arrows). At the times studied, the endothelium of the DA consists of a single cell layer; thus, since *tbx20* staining directly abuts the DA lumen but also extends greater than one cell diameter ventrally (Figure 1E, red arrow), under the influence of PMA both endothelial cells of the DA and the ventral mesenchyme beneath were induced to express *tbx20*. Thus, elevated Hh signaling can induce *tbx20* expression in the floor of the DA, making its absence there normally a likely consequence of distance from the notochord source of Hh (Figure 1A).

To examine HSC programming, we monitored *runx1* expression, which is essential for HSC development and is the first sign of HSC formation in zebrafish (Burns et al., 2005; Gering and Patient, 2005; Kalev-Zylinska et al., 2002). Despite the ectopic expression of *tbx20* in the DA floor, *runx1* expression in PMA-treated embryos was indistinguishable from control embryos at 24 hpf (Figures 1F and 1G, red arrows). Both the numbers of *runx1*$^+$ cells and their levels of expression were unaffected. There was a similar lack of effect at 23 hpf, when *runx1* expression in the DA is first detectable, and also at 25 and 26 hpf (data not shown). We therefore conclude that Hh affects neither the initiation of *runx1* expression in the DA nor its maintenance.

It was not possible to study expression of genes downstream of *runx1*, such as *cmyb* and *ikaros* (Gering and Patient, 2005; Thompson et al., 1998; Willett et al., 2001), since PMA-treated embryos do not develop circulation due to loss of the axial vein (data not shown), and therefore the primitive blood expressing *cmyb* and *ikaros* remains in the ICM region, masking expression in the HSCs in the DA floor. Furthermore, it was not possible to assess derivatives of the HSCs in the thymus, for example *rag1* expression (see Figures 2J, 2K, 4K, and 4L), because the absence of circulation prevents HSC migration to this region (Kissa et al., 2008; Murayama et al., 2006). However, the initiation and maintenance of an additional HSC marker, *gfi1*, was also unaffected in the DA of PMA-treated embryos, as seen for *runx1* (Figure S2). Taken together, the undisturbed initiation of *runx1* and *gfi1* expression in PMA-treated embryos indicates that distance from the Hh source cannot alone be responsible for the localization of HSC emergence in the DA floor.

Bmp Signaling Is Required for Initiation and Maintenance of *runx1* Expression in the Ventral Wall of the DA

Analysis of gene expression in the zebrafish trunk revealed that Bmp antagonists surround the pocket in which blood and endothelium differentiate, but that just prior to HSC emergence, the environment is transformed into one favoring Bmp signaling ventral to the DA (Figure 2A). Thus, the pocket in which the blood and endothelium differentiate is surrounded by tissues expressing *chordin*, *noggin2*, and *type II collagen* up to 22 hpf (Furthauer et al., 1999; Yan et al., 1995) (Figure S3). However, by 24 hpf, while *noggin1* remains expressed in the ventral somite distal to the DA, the pronephric ducts (PNDs) in the anterior, and the ventral mesenchyme in the posterior, express *bmp4* (Chin et al., 1997) (Figures S3A and S3B). In addition, the major vessels, including the DA, continue to express *tolloid* (from 21 hpf), which cleaves residual Chordin (Connors et al., 1999). Furthermore, *bmpr2a*, *alk1*, *alk3*, *alk8*, and *smad5* are all expressed in the DA (Monteiro et al., 2008). Thus, immediately prior to HSC emergence, the environment switches from anti- to pro-Bmp signaling, suggesting a potentially direct inductive role for Bmp in HSC emergence within the DA. Similar, localized expression of *bmp4* has been observed in human, mouse, chick, and *Xenopus* embryos (A. Ciau-Uitz and R.P., unpublished data; Marshall et al., 2000; Pimanda et al., 2007; Suonpaa et al., 2005).

To functionally test such a role for Bmp signaling, we used a transgenic zebrafish line expressing a temperature-inducible,

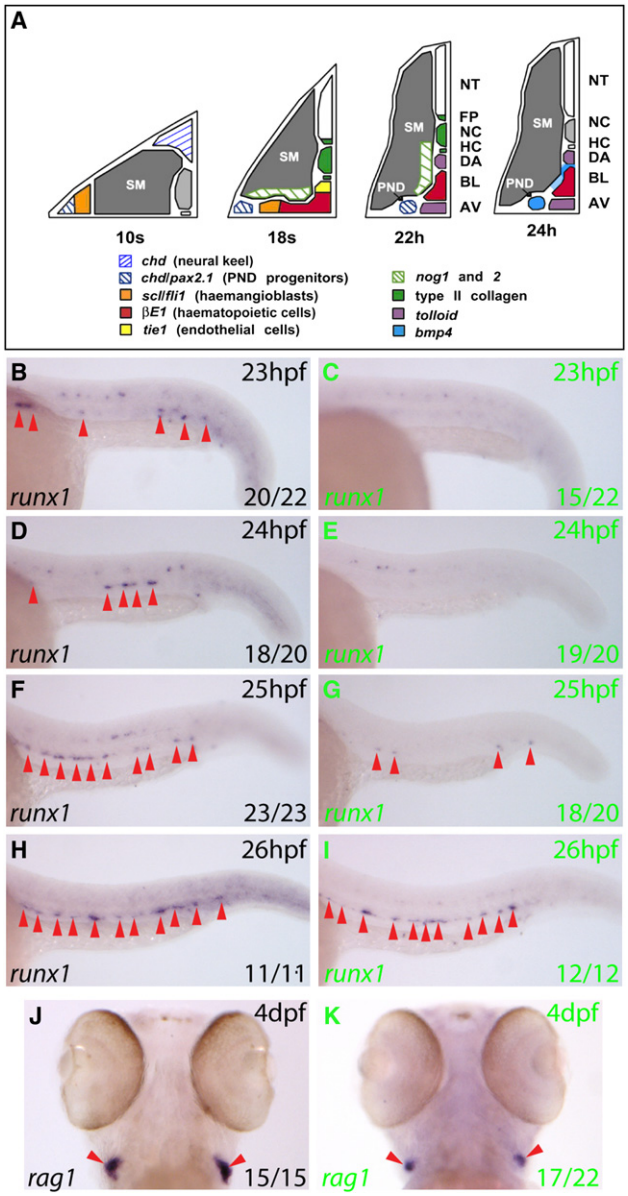

Figure 2. Bmp Signaling Is Required for Initiation of the HSC Program in the Ventral DA

(A) Gene expression summary as progenitors migrate to the midline and form blood and DA/AV endothelium. The DA region becomes conducive to Bmp signaling by 24 hpf.

(B–I) Expression of *runx1* (arrows) in embryos heat shocked at 21 hpf. *runx1* expression in the DA at 23–26 hpf (GFP$^-$, black letters) was substantially reduced in GFP$^+$ embryos (green letters) at 23–25 hpf. Representative embryos are depicted, and numbers with or without *runx1* staining were scored.

(J and K) *rag1* expression in thymi at 4 dpf heat shocked at 21 hpf. 17/22 GFP$^+$ embryos had reduced *rag1* expression compared to GFP$^-$ embryos, in which *rag1* staining showed little variation. The remaining 5/22 GFP$^+$ embryos exhibited wild-type *rag1* expression. AV, axial vein; BL, primitive blood; DA, dorsal aorta; HC, hypochord; NC, notochord; NT, neural tube; PND, pronephric duct; SM, somitic mesoderm.

truncated Bmp receptor (tBR), which acts as a dominant-negative mutant (Pyati et al., 2005, 2006). Because tBR is fused to GFP, fluorescent embryos expressing tBR can be separated after heat shock from control embryos derived from the same transgenic heterozygous parent. Embryos were heat shocked at 21 hpf so that Bmp signaling would be inhibited by 23 hpf, when *runx1* expression initiates in the DA. Immunohistochemistry for phosphorylated Bmp receptor Smads confirmed that Bmp signaling was inhibited by 23 hpf and recovering by 26 hpf (Figure S4).

Embryos fixed between 23 and 26 hpf were assayed for *runx1* expression (Figures 2B–2I). At 23 hpf, 20/22 GFP$^-$ embryos displayed *runx1*$^+$ cells in the DA, whereas 15/22 GFP$^+$ embryos possessed no DA staining. In both GFP$^+$ and GFP$^-$ embryos, *runx1* staining was detected equivalently in other areas of the embryo, such as the olfactory placode and the Rohon-Beard neurons, demonstrating that the loss of *runx1* expression was DA specific (Figure S5). The *runx1* reduction in the DA persisted to 25 hpf in the GFP$^+$ embryos, but by 26 hpf *runx1* expression was recovering (Figures 2H and 2I). Thus, the loss of *runx1* in the DA correlates with downregulation of Bmp signaling at 23–25 hpf, and recovery of *runx1* at 26 hpf correlates with the turnover of tBR and the recovery of phosphorylated Smad activity (Figure S4).

The first clearly identifiable derivatives of the definitive lineage in zebrafish embryos are the *rag1/2*- and *ikaros*-expressing thymocytes in the bilateral thymi at 4 dpf (Kissa et al., 2008; Willett et al., 1997), and we could test for the presence of these cells since all embryos possessed normal circulation. Consistent with a loss of HSCs in the DA, *rag1* expression in the same batch of embryos at 4 dpf was reduced in GFP$^+$ embryos compared with GFP$^-$ embryos (Figures 2J and 2K). Furthermore, although analysis of additional HSC markers such as *cmyb*, *ikaros*, or *cd41* was not possible since these genes are expressed in the overlying primitive blood at the time of HSC emergence (Gering and Patient, 2005; Lin et al., 2005; Thompson et al., 1998), the expression of *gfi1*, which is restricted to the definitive lineage, behaved in an identical manner to *runx1* following Bmp inhibition (Figure S6). Taken together, these results demonstrate that Bmp signaling is required for the initiation of the HSC program in the DA and for normal levels of HSC descendants.

To determine if Bmp signaling is required for maintenance as well as initiation of the definitive hematopoietic program, we performed a heat shock at 22 hpf and assayed for *runx1* expression (Figures 3A–3H). GFP$^+$ (24/24) and GFP$^-$ (19/19) embryos both possessed strong *runx1* staining in the DA at 23 hpf. However, at 24 hpf, a strong downregulation of *runx1* expression was observed in GFP+ embryos, which persisted until 26 hpf. These results demonstrate that Bmp signaling is required both for the initiation and maintenance of *runx1* expression in the floor of the DA.

To determine if elevated Bmp signaling could expand the domain of *runx1* expression dorsally, we analyzed the expression of *runx1* and *gfi1* in *dino* mutants, which possess a genetic lesion at the *chordin* locus (Figure S7) (Schulte-Merker et al., 1997). Surprisingly, the initiation and maintenance of both markers in the DA were unaffected in *dino* embryos, and neither gene exhibited ectopic expression in the DA (Figures S7A–S7J, red arrows); however, both were ectopically expressed in the posterior ICM as previously demonstrated (Leung et al., 2005). Arterial expression of *tbx20* was unaffected, while its expression within a ventral population of cells under the yolk cell

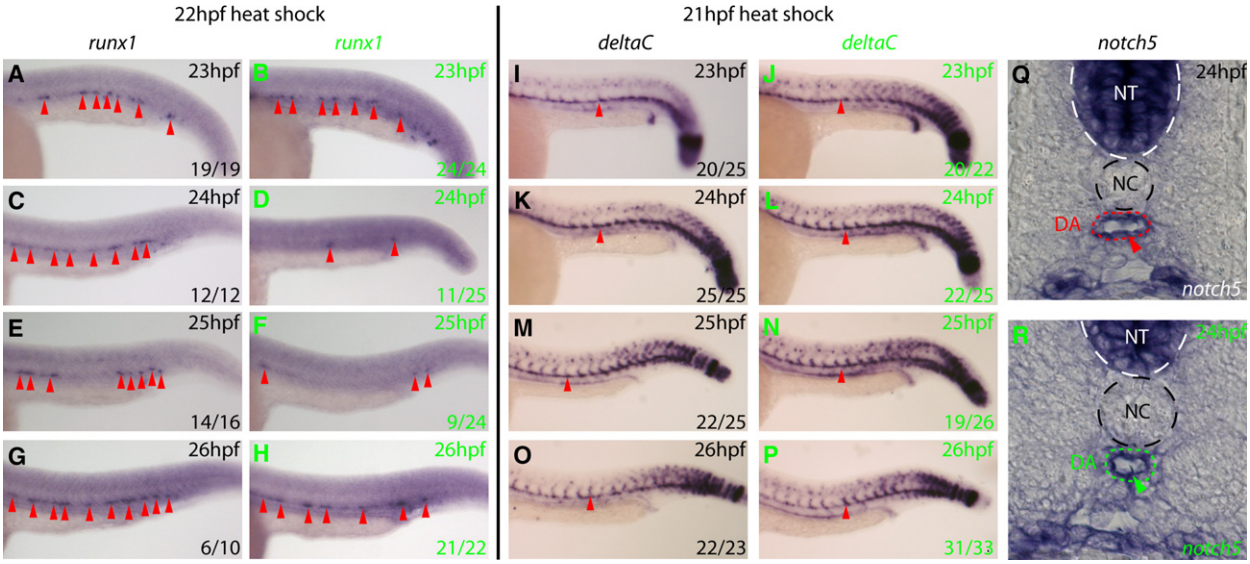

Figure 3. Bmp Signaling Is Required for Maintenance of *runx1* Expression in the Ventral DA but Not for Maintenance of the Arterial Program
(A–H) *runx1* expression (arrows) in embryos heat shocked at 22 hpf. *runx1* initiated normally, was lost by 24 hpf in GFP$^+$ embryos (green letters) compared to GFP$^-$ embryos (black letters), but recovered by 26 hpf. Representative embryos are shown. Embryos containing a few *runx1*$^+$ cells were scored as negative.
(I–P) *deltaC* expression in the DA (arrows) in embryos heat shocked at 21 hpf was unaffected by inhibition of Bmp signaling.
(Q and R) *notch5* expression in the ventral wall of the DA (arrow) in sectioned embryos heat shocked at 21 hpf was normal.

extension was substantially increased in *dino* mutants in comparison to wild-type embryos (Figures S7K and S7L, yellow and orange arrows, respectively), as expected for Bmp-dependent expression (Figures S8A–S8H, red arrows) (Pyati et al., 2006). Preliminary experiments with a heat-shock-inducible Bmp2b transgenic zebrafish line (Chocron et al., 2007) support this lack of HSC gene expression in the dorsal DA (data not shown). We therefore conclude that Bmp levels may already be optimal ventrally and that dorsal cells may be unable to respond.

The Arterial Program Is Unaffected by Loss of Bmp Signaling

Since arterial specification and HSC emergence are intimately linked (Burns et al., 2005; Gering and Patient, 2005), the loss of *runx1* in the DA led us to examine the arterial program in Bmp-inhibited embryos. Arterial markers such as *notch5* and *deltaC* are expressed throughout the DA roof and floor from 22 hpf, shortly before *runx1* expression (Patterson et al., 2005). We therefore induced tBR expression via a 21 hpf heat shock and assayed arterial gene expression alongside *runx1*. We found no difference between GFP$^+$ and GFP$^-$ embryos at any stage, even at stages when *runx1* expression was lost (Figures 3I–3R, and data not shown). Thus, the loss of *runx1* expression was not due to a loss of endothelium in the DA floor, but a loss of expression within those cells. Analysis of *tbx20* expression also revealed no differences between the GFP$^+$ and GFP$^-$ embryos in expression or polarization of *tbx20* transcripts (Figure S8). Taken together, these observations demonstrate that Bmp signaling within the trunk prior to the onset of HSC gene expression is required for initiation of definitive hematopoiesis in the ventral wall of the DA but not the maintenance of ventral arterial marker expression in those cells.

bmp4 Is Required for HSC Emergence within the Ventral DA

Since induction of tBR produced only transient inhibition of Bmp signaling (Figure S4), which led to recovery of HSC gene expression (Figures 2B–2I) and consequently only a moderate downregulation of *rag1* at 4 dpf (Figures 2J and 2K), we wished to determine if a more complete loss of Bmp signaling could create a more profound effect on the HSC program. Given the presence of *bmp4* transcripts proximal to the DA floor during the initiation of definitive hematopoiesis in zebrafish (Figure 2A and Figures S3A and S3B), and also in other vertebrates, together with the absence of *bmp2b* and *bmp7* transcripts in zebrafish (data not shown), we knocked down *bmp4* expression by morpholino (MO) injection (Chocron et al., 2007). Embryos injected with *bmp4* morpholino displayed inappropriate expression of *gata1* at 10S in the most ventral posterior mesoderm (Figures S9A and S9B), as previously demonstrated in a *bmp4* mutant (Stickney et al., 2007). At 26 hpf, *bmp4* morphants exhibited complete loss of *runx1* expression within the DA (Figures S10A and S10B); however, neural *runx1* expression remained unaffected, demonstrating that *bmp4* is required for the initiation of *runx1* expression in the ventral wall of the DA. Furthermore, at 28 hpf, *runx1* expression remained absent from the DA in two-thirds of *bmp4* morphants (Figures 4A–4D, arrows), while *cmyb* expression was also substantially downregulated in the DA of *bmp4* morphants (Figures 4E–4H, red arrows). Interestingly, *cmyb* expression within the erythromyeloid progenitors (EMPs) in the posterior blood island was unaffected in *bmp4* morphants (Figures 4G and 4H, green arrows), supporting previous studies which indicate that EMPs arise independently of HSCs (Bertrand et al., 2007). While HSC gene expression was reduced, the expression of arterial markers was unaffected in *bmp4* morphants (Figures S9C–S9J), showing that the *bmp4* signal acts

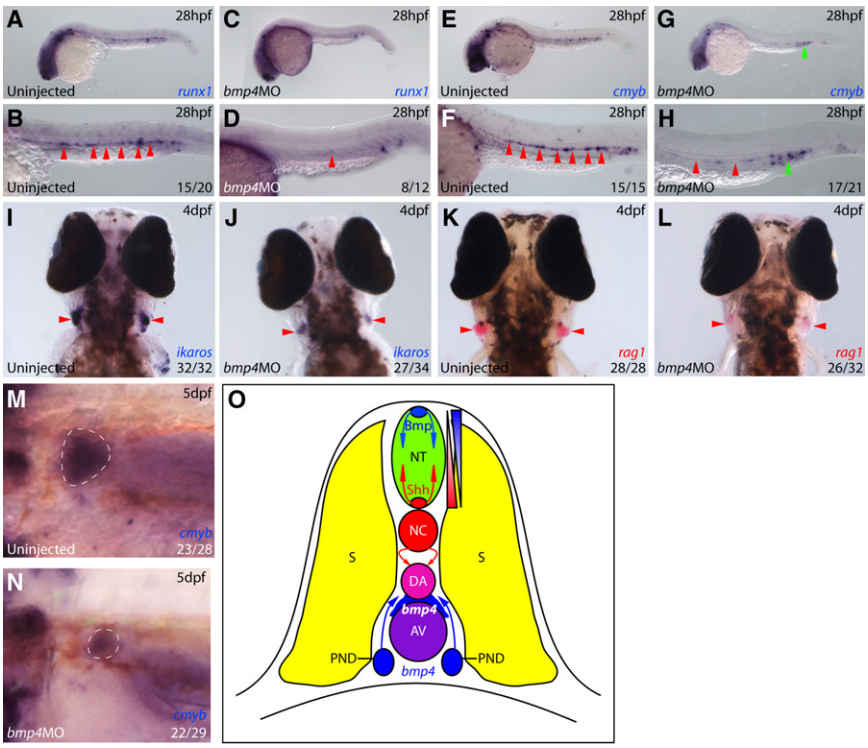

Figure 4. *bmp4* Is Required for HSC Emergence within the Ventral DA

(A–D) *runx1* expression was absent from the DA (arrows) in 8/12 *bmp4* morphants at 28 hpf (C and D), while the remaining 4/12 exhibited reduced expression compared with uninjected controls (A and B).

(E–H) At 28 hpf, 17/21 *bmp4* morphants exhibited absent or substantially downregulated *cmyb* expression in the DA (red arrows).

(I and J) At 4 dpf, 27/34 *bmp4* morphants exhibited substantial downregulation of *ikaros* expression within the developing thymi (arrows). The remaining 7/34 exhibited wild-type expression.

(K and L) At 4 dpf, 26/32 *bmp4* morphants exhibited substantially reduced expression of *rag1* in developing thymi (arrows), of which 7 exhibited no *rag1* expression. The remaining 6/32 were wild-type.

(M and N) Dorsolateral views of the developing pronephros at 5 dpf depicting substantial downregulation of *cmyb* expression (outlined) in 22/29 embryos, of which 4/29 exhibited no expression. The remaining 7/29 were wild-type.

(O) Diagram of zebrafish trunk depicting polarization of the DA by dorsal Hedgehog and ventral Bmp signaling, mirroring the neural tube.

specifically on the definitive hematopoietic program and not the maintenance of arterial gene expression within the DA.

To confirm that the morpholino-induced loss of *runx1* expression in the DA truly reflected a loss of HSCs, we assayed *rag1*$^+$, *ikaros*$^+$ thymocytes at 4 dpf. *bmp4* morphants contained substantially reduced *rag1* and *ikaros* expression within the thymus, while expression of *foxn1* in the thymic epithelium was unaffected, demonstrating that the thymi were present but contained substantially fewer thymocytes (Figures 4I–4L, and Figures S10C–S10F, arrows). To confirm that HSCs and not just T cells were affected, we analyzed the expression of *cmyb* within the developing kidney at 5 dpf and found that *bmp4* morphants exhibited a substantial downregulation or total absence of *cmyb* in comparison to controls (Figures 4M and 4N, outlined). Taken together with the downregulation of *rag1* observed in embryos expressing tBR (Figures 2K and 2L), these data indicate that *bmp4* induces HSCs in the DA floor. Thus, overall, the data presented here show that dorsoventral patterning of the DA is a mirror image of the patterning of the neural tube, whereby Bmp specifies ventral rather than dorsal cell fates and Hh specifies dorsal rather than ventral cell fates (Figure 4O).

DISCUSSION

The first HSCs in developing embryos are clearly located in the floor of the DA; however, whether they arise from the wall of the DA, the underlying mesenchyme, or the embryonic blood has been a matter for some considerable debate (Dzierzak and Speck, 2008). However, evidence supporting an endothelial precursor for the HSC continues to accumulate, most recently using VE-Cadherin to label the cells (Chen et al., 2009; Zovein et al., 2008). Furthermore, both cell lineage tracing and genetic disruption of signaling pathways support independent development of embryonic blood and HSCs, and the shared signaling dependence of the arterial and HSC programs also support the DA wall as the source of the HSCs (Ciau-Uitz et al., 2000; Burns et al., 2005; Gering and Patient, 2005). We have now identified two signals that differentially affect these two programs, with Hh maintaining the dorsal arterial program and Bmp inducing the ventral HSC program.

In chick embryos, at the time of HSC emergence, the DA contains two different mesodermal contributions, with the floor derived from splanchnopleural mesoderm and the roof from somites (Pouget et al., 2006). It is formally possible, therefore, that the observed polarization of the DA, with HSCs only arising in the floor, reflects cell lineage rather than local signaling. However, thus far in zebrafish, evidence has only been produced for a contribution to the DA from lateral (splanchnopleural) mesoderm (Zhong et al., 2001). In addition, more recent evidence from the chick indicates that even the roof of the DA initially derives from splanchnopleural mesoderm (T. Jaffredo, personal communication; Pouget et al., 2006). Thus, even in the chick the splanchnopleural mesoderm-derived DA is likely to be patterned before its replacement by somitic endothelial cells. However, the failure to induce ectopic HSC gene expression in *dino* embryos suggests that Bmp signaling may be optimal around the ventral DA and that the dorsal wall of the DA may not be competent to receive Bmp signaling.

The precocious differentiation of the posterior PLM into *gata1*$^+$ primitive blood induced by *tbx20* knockdown can be mimicked by morpholino knockdown of *bmp4* (Figures S9A–S9B), since *tbx20* is under the control of Bmp signaling in these cells (Pyati et al., 2006). Conversely, in *dino* mutant embryos, which exhibit increased Bmp signaling, *tbx20* expression is expanded in the

posterior PLM and *gata1* expression is reduced (data not shown). Thus, *bmp4* controls the timing of primitive hematopoietic differentiation in the posterior PLM via *tbx20*. However, since Hh-dependent *tbx20* expression in the DA does not antagonize definitive hematopoiesis, its presence there likely reflects its role in vascular lumenization and intersomitic vessel sprouting (Szeto et al., 2002). Furthermore, in contrast to its role in controlling the timing of primitive hematopoietic differentiation in the posterior PLM, *bmp4* expression around the DA is required to promote definitive hematopoietic specification by initiating the expression of *runx1* in the ventral wall of the DA.

Requirements for Notch and Hh signaling have been demonstrated for the expression of *runx1* within the DA, albeit at different times (Burns et al., 2005; Gering and Patient, 2005). While a direct role for Hh signaling remains to be demonstrated, Notch signaling has been shown to be required directly for HSC gene expression in the DA at 36 hpf (Burns et al., 2005). However, since we and others have shown that HSC gene expression is observed within the DA much earlier than this (Gering and Patient, 2005; Kalev-Zylinska et al., 2002), it remains to be determined whether Notch signaling acts directly in the initiation of definitive hematopoiesis within the DA. Therefore, it is formally possible that, while *bmp4* is responsible for initiation and early maintenance of the HSC program, Notch signaling may be important in the later maintenance of the program; however, parallel inputs of both pathways cannot be excluded.

The model proposed here for zebrafish seems likely to apply to other vertebrates, because *bmp4* is expressed ventral to the DA in human, mouse, chick, and *Xenopus* embryos (A. Ciau-Uitz and R.P., unpublished data; Marshall et al., 2000; Pimanda et al., 2007; Suonpaa et al., 2005). Furthermore, Bmp receptor Smads have been shown to transactivate the *runx1* promoter in a mouse myeloid cell line (Pimanda et al., 2007). Moreover, addition of Bmp4 to mouse AGM explants increases transplantable hematopoietic activity, although since in these experiments HSCs had already been formed, it was difficult to exclude an effect on expansion, as demonstrated for purified human hematopoietic progenitors (Bhardwaj et al., 2001; Durand et al., 2007). Thus, to the best of our knowledge, our studies in developing zebrafish embryos represent the first demonstration that Bmp is required for HSC formation in vivo. Together with the observation that Hh is required for maintenance of the arterial program in the dorsal wall of the DA, the Bmp-dependent emergence of HSCs ventrally in the DA indicates that patterning of the DA has parallels with that of the neural tube (Figure 4O).

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry

Zebrafish (*Danio rerio*) embryos were obtained from a wild-type strain and raised at 28.5°C (Westerfield, 1993). Hemizygous transgenic embryos expressing under heat shock control a dominant-negative Bmp receptor fused to GFP *Tg(hsp70l:dnBmpr-GFP)* or Bmp2b *Tg(hsp70l:bmp2b)* were used (Chocron et al., 2007; Pyati et al., 2005).

Heat Shock Inductions

Batches of transgenic embryos were strictly staged as described (Kimmel et al., 1995) to ensure minimal developmental asynchrony and subjected to a 43°C heat shock for 30 min at 21 or 22 hpf as described (Pyati et al., 2005).

Purmorphamine Treatments

Purmorphamine (Chemistry Research Laboratory, South Parks Rd, Oxford, UK; stock solution in DMSO: 2.5 mg/ml) was used in aquarium water at 20 μM. Embryos were treated from before MBT and from the 15-somite stage until collection.

In Situ Hybridization

In situ hybridization was performed as described (Gering and Patient, 2005). RNA probes were labeled with digoxigenin (Roche) and detected using BM Purple (Roche).

Wax Sectioning

Embryos were sectioned in paraffin wax (Walmsley et al., 2002).

Antibody Staining of Phosphorylated Smad1, 5, 8

Embryos were fixed in 4% PFA in phosphate buffered saline (PBS) at 4°C and washed in ethanol, and endogenous peroxidase activity was blocked by incubation in 0.5% normal goat serum (NGS) (Sigma)/0.5% H_2O_2 for 30 min at 25°C before blocking in 10% NGS in PBS Tween (Sigma) for 2 hr at 25°C. Incubation with pSmad1/5/8 antibody (Cell Signaling Technology) (1:200) was overnight at 4°C. Incubation with secondary antibody (goat α-rabbit; HRP 1:200) (Vectorlabs) was for 2 hr at 25°C. Staining was developed using 3, 3′-diaminobenzidine (DAB) (Vectorlabs SK4100) as per the manufacturer's instructions. Embryos were refixed at 4°C in 4% PFA.

bmp4 MO Injections

Injections were performed with 0.5 nl *bmp4* morpholino (2 ng/nl dissolved in distilled water) (Gene Tools) 5′-GGTGTTTGATTGTCTGACCTTCATG-3′ (Chocron et al., 2007).

SUPPLEMENTAL DATA

Supplemental Data include nine figures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00176-2.

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